EVALUATING THE EFFECTS OF WATER HYACINTH SUBSTRATES UPON THE GROWTH OF YEAST OF THE GENUS CANDIDA FOR THE PRODUCTION OF "SINGLE CELL PROTEIN."

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ABSTRACT

An abundantly growing water plant in tropical and subtropical areas called water hyacinth (Eichhornia crassipes) is the main topic of this study. An analysis of this water plant has indicated a spectrum of crude proteins (13-19 %), lipids (1 %) and carbohydrates (0.85-1.3 %). Its plant juice when released by osmosis using different concentrations of sodium chloride was found to be unable to support the growth of Candida tropicalis owing to low carbohydrate and nitrogen levels. However, when its plant juice was prepared by homogenation (with distilled water as diluent) and heating for half an hour at 110^0 C, its carbohydrate and nitrogen levels were sufficient to support the growth of the organisms. A dry yield of yeast biomass recovered from plant juice was highly comparable to that recovered from standard Sabouraud Dextrose Broth, a highly refined nutrient commonly used for the propagation of above organisms.

In order to utilize this plant to the fullest, its residues were used as a substrate for methanogenic bacteria which were unable to tolerate even very low sodium chloride concentrations. The result was encouraging, however, though the time between inoculation and the earliest production of methane gas was somewhat delayed.

Further studies revealed that it does not have antibacterial activity and is incapable of absorbing bacteria or viruses. Its oxalic acid concentration (0.394 %) is assumed to be safe enough for the consumption of the plants by cattle and poultry.

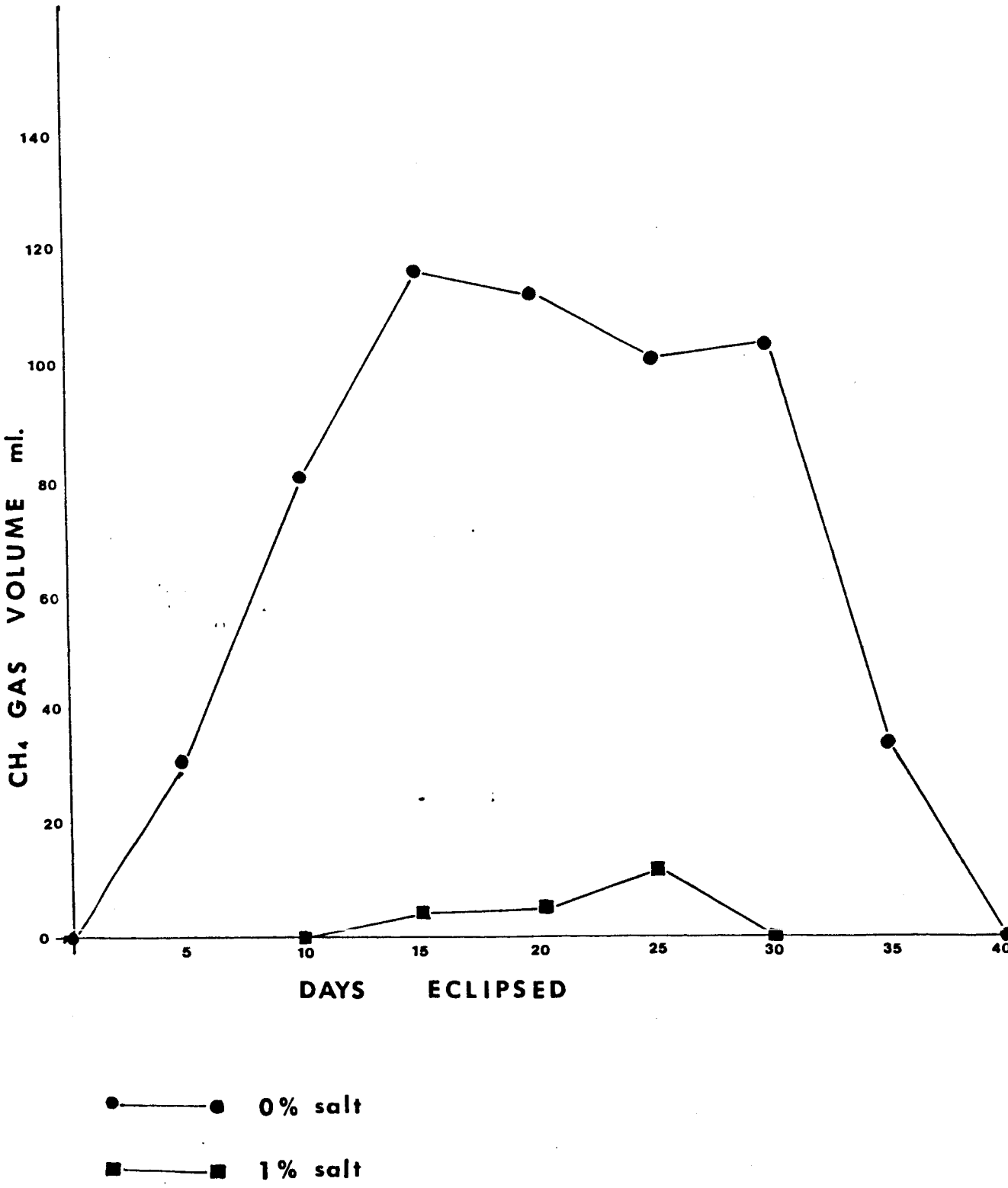
1. Salt Tolerance of Methanogenic Bacteria

Anaerobic digestion is usually considered to be a two-stage process consisting of acid formation (liquefication) and gas formation (gasification). Since not only acids are produced as metabolic end-products during the first stage, and not all the gas formed during anaerobic digestion is derived from the second stage, acid formation should therefore be named the non-methanogenic phase and the gas-formation should be the methanogenic phase. Bacteria reported to be involved in the non-methanogenic phase included Aerobacter, Aeromonas, Alcaligenes, Bacillus, Bacteroides, Clostridium, Escherichia, Klebsiella, and many more. In addition to bacteria, others like protozoa, fungi and yeasts are also taking part in the digestion process. These microorganisms mainly convert lipids, proteins and carbohydrates to short-chain organic acids, alcohols, ketones and fatty acids.

There are mainly three different types of anaerobic bacteria responsible for the methanogenic phases. These are Methanosarcina, Methanococcus and Methanobacterium. They are able to break down the short-chain organic acids, alcohols, ketones and fatty acids into methane gas and carbon dioxide.

It was found that the methanogenic bacteria were practically intolerant to even a relatively low degree of NaCl concentrations as shown in Fig. 1. Plant residues soaked in 1 % NaCl evolved less than 20 ml of methane gas during the observation period, as compared to the control (0 % salt) which could evolve more than 600 ml of methane gas. Plant residues soaked in 3 %, 5 %, 7 %, 9 %, 11 %, 13 % and 15 % of NaCl concentrations could not evolve any methane gas. Methanogenic bacteria in these concentrations

Figure 1. Salt Tolerance of Methanogenic Bacteria

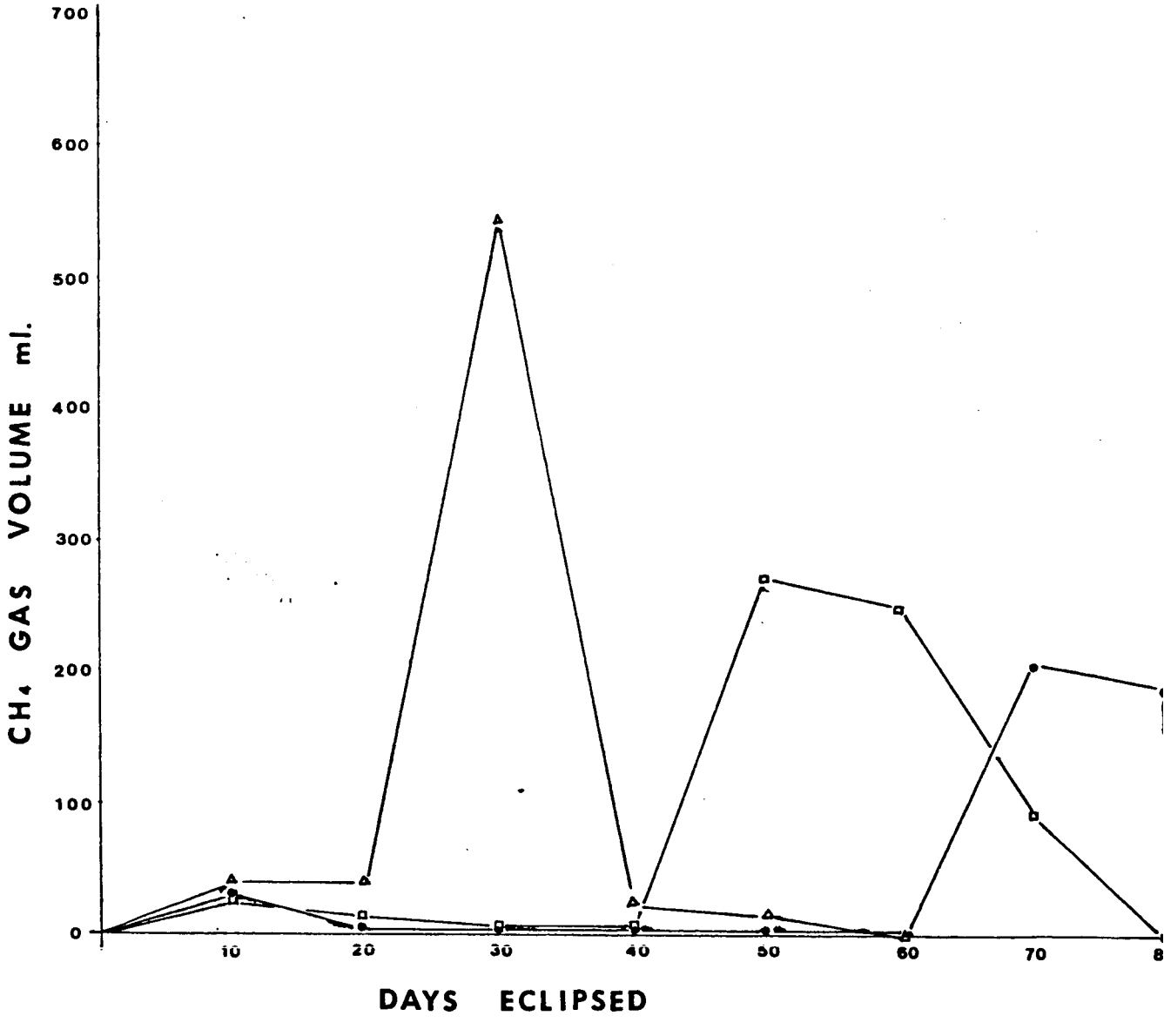

140
120
100
80
60
40
20
0
CH_4 GAS VOLUME ml.
5
10
15
20
25
30
35
40
DAYS ECLIPSED
0% salt
1% salt

were dead. Since many bacteria, fungi, and yeasts involved in the non-methanogenic phase are salt tolerant, it is hard to believe they are unable to break down nutrients (carbohydrates, lipids and proteins) to provide energy for the methanogenic bacteria. There are three theories to explain the phenomenon observed in this study. It is possible that the metabolic end-products (short chain organic acids, alcohols, ketones and fatty acids) produced by non-methanogenic microorganisms are exhausted by other types of microorganisms that can utilize these end-products. Secondly, the possibility exists that there are a number of various substrates to be utilized for gas production. However, they can not be acted upon unless three growth factors are present. These factors are: 1. large amount of acetate, 2. a volatile acid (extracted by ether at pH 2, having a distillation rate similar to butyric acid or long-chain fatty acids), 3. a not absolutely identifiable factor which was found to be dialysable and was not inactivated by autoclaving at 121^{0} C in a 0.86 M HCl solution at a pH of 2. A disappearance of either one of these three factors will result in the cessation of the methane gas production. The third possibility is that methanogenic bacteria can not endure any salt concentration higher than 1 %.

2. Methanogenic Bacteria Growing in Plant Residues

Methanogenic bacteria growing in plant residues which had not previously been soaked in any degree of salt concentration produced more than 800 ml/250 g of methane gas (Fig. 2). The highest volume produced was between 20 and 40 days. The production declined rapidly and came to completion after two months.

Figure 2. Methanogenic Bacteria Growing in Plant Residues



△——△ Plant tissue treated with 0% salt

●——● " 5% "

□——□ " 3% "

Methanogenic bacteria growing in plant residues which had been previously soaked in 3 % NaCl concentration and washed repeatedly in distilled water, produced less than 50 ml/250 g of methane gas in the first forty days. However, about 500 ml/250 g of methane gas were produced in the next forty days. Gas ceased to evolve on the 80th day.

For those methanogenic bacteria growing in plant residues which had been previously soaked in a 5 % NaCl concentration to extract most of its plant juice exhibited more or less the same kind of gas production pattern as those plant tissues soaked in a 3 % NaCl concentration. The only difference was that it started producing a large amount of methane gas on the 70th day and stopped twenty days later. The total volume was about 500 ml/250 g.

When plant tissues were soaked in 3 % and 5 % salt solution, respectively, many of the free nutrients were extracted out. Therefore, a deficiency of nutrients existed within the residue. However, as the period of incubation carried on, cellulose, bound proteins and lipids were broken down by non-methanogenic microorganisms. This breakdown led to the formation of simple acids, alcohols, fatty acids and ketones which served as the substrates of methanogenic bacteria. It is concluded that this was the reason why methane gas did not begin to evolve until the 40th day (3 %) and 60th day (5 %).

3. Carbohydrates, Lipids and Protein Contents in Water Hyacinth

a. Total extractable crude proteins was calculated as follows:

Total 0.5 N NaOH added---------- X ml

Total dry weight of water hyacinth---------- W g

Absorbance of standard bovine albumin every

0.2 mg/ml at 650 nm---------- Z

Absorbance of sample every 2 ml at 650 nm--------- Y

$$\text{Per cent protein of water hyacinth by dry weight} = \frac{\frac{Y}{Z} \times 0.2 \times X}{1000 \text{ mg/g}} \times \frac{1}{W} \times 100\%$$

The crude proteins of water hyacinth was found to be in the range of 13-19 %.

b. The extractable carbohydrates were calculated as follows:

Total TCA added---------- B ml

Total dry weight of water hyacinth---------- C g

Absorbance of standard glucose solution

0.1 mg/2 ml at 620 nm---------- D

Absorbance of samples every 2 ml at 620 nm-------- E

$$\text{Per cent of total extractable sugars of water hyacinth by dry weight} = \frac{\frac{E}{D} \times 0.1 \times B}{1000 \text{ mg/g}} \times \frac{1}{C} \times 100\%$$

The percentage of extractable total sugars in water hyacinth was found to be 0.85-1.307 % with an average of 0.8905 %.

c. Total extractable lipids was calculated as follows:

$$\text{Per cent of total extractable lipids} = \frac{\text{weight of lipids}}{\text{dry wt. of water hyacinth}} \times 100\%$$

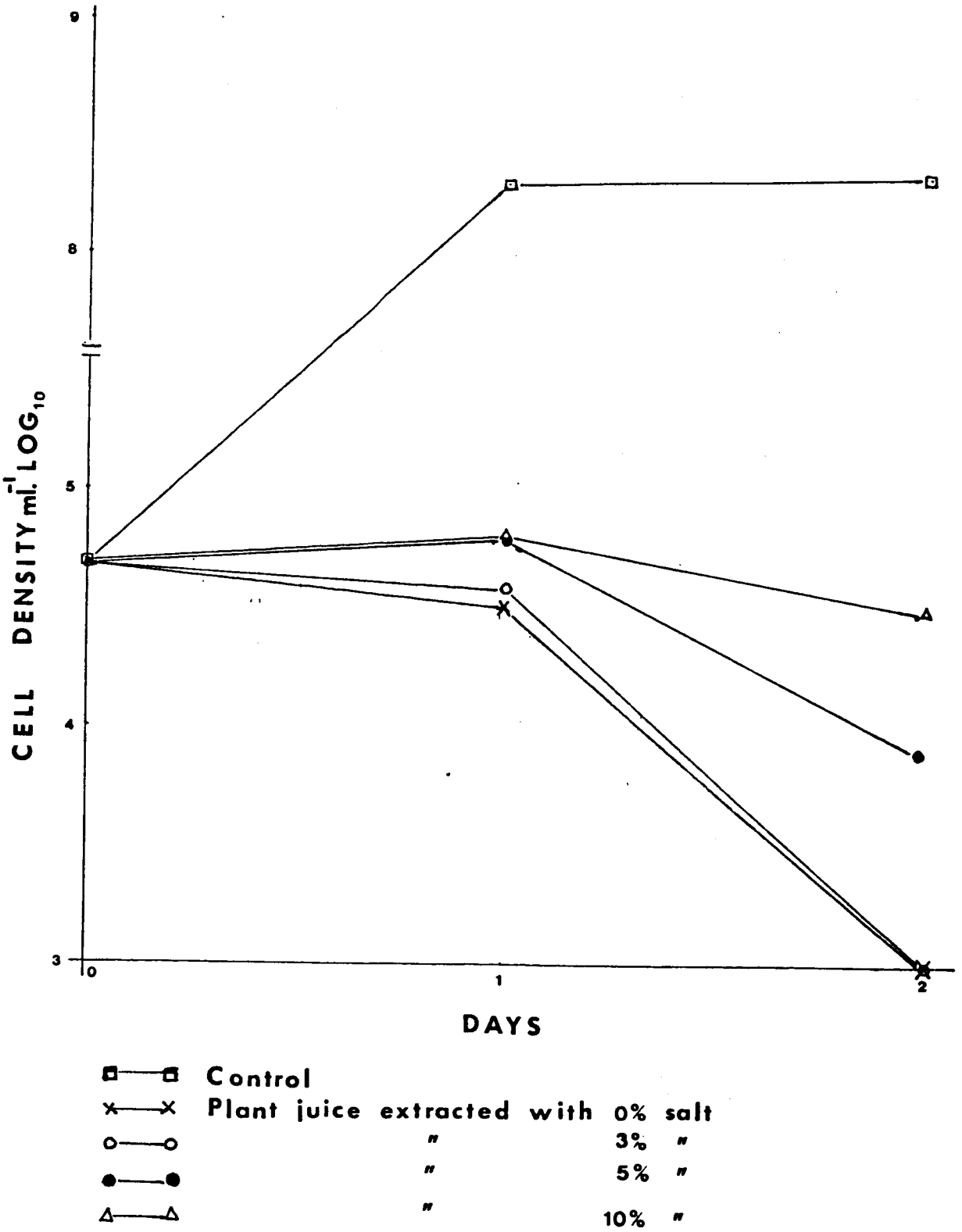
The extractable lipids of water hyacinth was found to be about 1 %.

Boyd reported that water hyacinth contained 11-12 % protein, 1.5-2.5 % lipids and 8 % carbohydrates by dry weight. The variations in the percentage of protein, carbohydrates and lipids may be due to methods of extraction and age of the plants because the more matured plants should have higher levels of protein, carbohydrates and lipids.

4. Growth of Candida tropicalis in Salt Extracted Plant Juice

C. tropicalis expresses no growth in plant juices extracted by various salt concentrations (3 %, 5 %, 10 %) as shown in Fig. 3. Inversely, they died gradually. However, yeast growing in the control medium (Sabouraud Dextrose Broth) revealed a usual growth pattern. When plant juices extracted by different concentrations of sodium chloride were tested for their sugar and nitrogen levels, it was discovered that both of them were very low, about 0.1-0.2 % and 0.04-0.2 %, respectively. These concentrations of carbohydrate and nitrogen were not enough to support the growth of C. tropicalis. This was also revealed by a gradual decline in the number of total cells.

Figure 3. Growth of Candida tropicalis in Salt Extracted Plant Juice


9
8
5
4
3
0
1
2
CELL DENSITY ml.$^{-1}$ LOG$_{10}$
DAYS
Control
Plant juice extracted with 0% salt
" 3% "
" 5% "
" 10% "

5. Growth of Candida tropicalis in Plant Juice Homogenate

The carbohydrate level found in the plant juice homogenate was approximately 0.85-1.3 % while the nitrogen level was about 0.8-1.2 %. It was also found that if the homogenate was heated at 110° C for 30 minutes a better yield of carbohydrate was recorded. According to Molina, when the pH of the homogenate was adjusted to a pH of 13 and heated at 70° C for one hour, an increase in protein concentration would be observed in jack-beans. With water hyacinths only a slight increase of 0.4 % in its protein concentration was indicated.

On the first 24 hours of incubation, cell density in flask 1 (plain plant juice), flask 4 (plant juice, 1 % masonex, 1% ammonium sulfate) and flask 5 (control) increased tremendously, about $4x10^3$ folds. Flask 2 (plant juice, 1 % ammonium sulfate) and flask 3 (plant juice, 1 % masonex) only increased to $1x10^3$ folds and $2x10^3$ folds respectively (Fig. 4). On the second and third day, cell density declined rapidly except in the control. The reasons for the decrease of viable cells might have been caused by an alkaline pH in the experimental flasks, nutrient depletion, accumulation of toxic products (metabolites), etc. The pH in the experimental medium increased from an initial pH of 5.6 to a pH of 8.5. The control group, however, maintained a pH of 5.6. A careful analysis for the reason of an increase of pH in the growth curve study of C. tropicalis growing in plain plant juice revealed that the pH increased from 5.60 to 6.5 within 12 hours (Fig. 5). Two hours later, a pH of 8.5 was measured. The pH of the control (SDB) was unchanged, remaining at 5.60. Among possible reasons responsible for the decline of viable cells, the most probable cause was the adverse pH since the optimum pH for C. tropicalis was 5.0-5.50.

Figure 4. Growth of Candida tropicalis in Plant Juice Homogenate

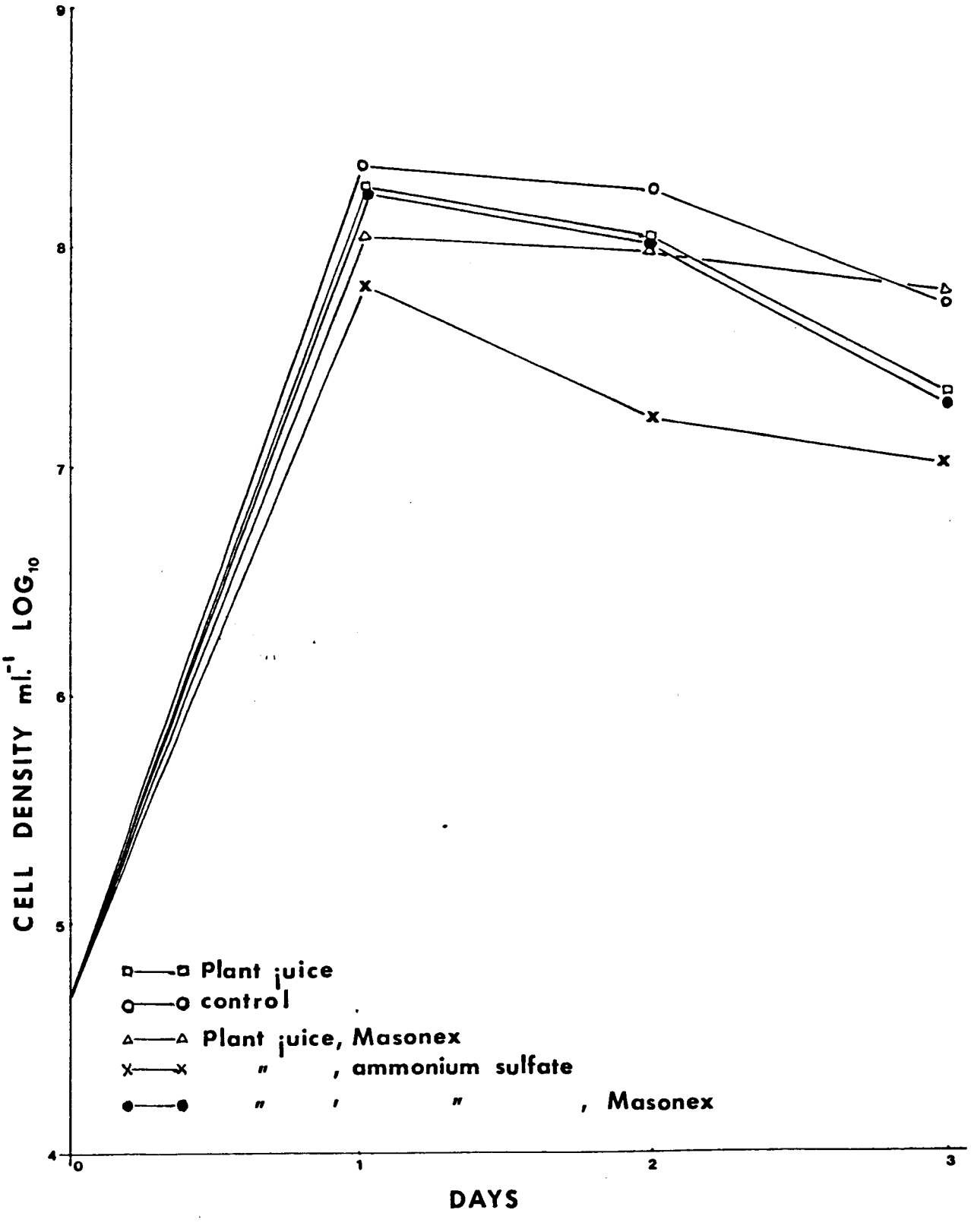
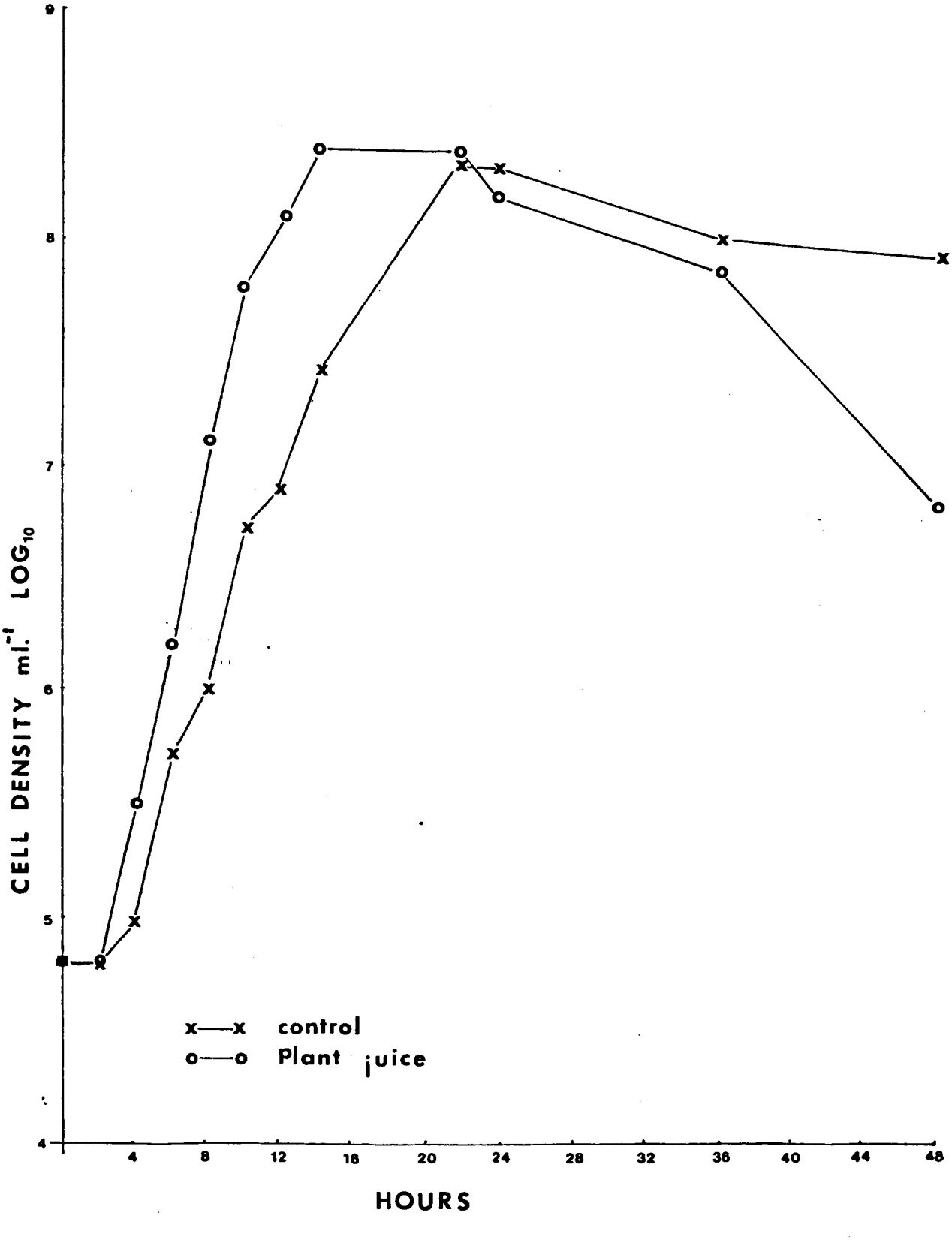

9
8
7
6
5
4
0
1
2
3
CELL DENSITY ml.$^{-1}$ LOG$_{10}$
DAYS
Plant juice
control
Plant juice, Masonex
" , ammonium sulfate
" " , Masonex

Figure 5. Growth Curve of C. tropicalis Growing in Plant Juice


CELL DENSITY ml.$^{-1}$ LOG$_{10}$
9
8
7
6
5
4
4
8
12
16
20
24
28
32
36
40
44
48
HOURS
x—x control
o—o Plant juice

The build-up of pH might be due to a lack of buffer systems in the plant juice mixture. Attempts had been made to maintain the pH at 5.60. These included the adjustment of the pH by addition of HCl and use of phosphate buffers. Further studies are indicated to determine the rapid change of the pH toward the alkaline side.

Since the plain plant juice was capable of yielding as many yeast cells as the SDB control broth, it was unnecessary to add other supplementary nutrients, such as ammonium sulfate and masonex, to the plant juice. One thing worth noticing was the fact that yeast cells growing in plant juice had a smaller cell size and an elongated shape, as compared to those growing in the Sabouraud Dextrose broth control, which revealed a larger size and a spherical shape. In addition, they revealed a different colonial morphology when grown on plant juice agar (1.5 % agar was added to the plant juice). Although the exact mechanisms which govern the cell shape and size escape a definite answer, Henning suggested that cell shape might be resulting from the influence of surface tension on a fluid surface, determined by specific genetic information, a direct consequence of the internal pressure of the cell, or nutritional state of medium. Magasanik also discovered that a lack of thymine resulted in a high frequency of mutation which undoubtedly would lead to a different cell shape and size.

A growth curve study of C. tropicalis growing in plant juice indicated that it established its highest cell density at 12-14 hours after incubation (Fig. 5). However, when growing in SDB it would not reach its highest cell density until 22 hours after incubation. A shorter time required by yeast cells growing in plant juice to attain their highest density might be due to the induction of particular enzymes to utilize the available energy source in plant juice.

Cells growing in plant juice declined rapidly after maintaining 8 hours of stationary phase. However, the decline of cells growing in SDB was not so drastic. This might be due to the exhaustion of energy sources, build-up of pH, or some other factors as described previously.

6. Yield of Candida tropicalis by Dry Weight

The yield of yeast biomass growing in 500 ml of a 1:1 dilution of plant juice by dry weight was 70 % when compared to the yeast biomass growing in 500 ml of regular concentration SDB in the first 24 hours. However, after an incubation period of 48 hours, the comparative yield of biomass dropped to 61 % (Table I and II), due mainly to the exhaustion of nutrients and the smaller cell size of the yeast cells growing in the plant juice. Oyakawa reported that when this growing process was carried out in large scale and continuous cultivation, the comparative yield of yeast biomass (growing in plant juice) could be increased to as high as 170 %. Gradova and his associates also pointed out that intensive biomass production might be achieved only by means of continuous processes.

In another experiment, yeast cells were inoculated into non-diluted plant juice in hopes that it would yield a higher harvest. However, the cell density increased only slightly, when compared to the diluted plant juice.

Table I

Comparison Of Dry Weight Of Yeast Cell Mass After 24 Hours Of Incubation In Sabouraud Dextrose Broth And Plant Juice

Sample	Cell Density $1x10^6$		Dry Weight of Cell Mass (g)	
	Control	Plant Juice	Control	Plant Juice
1	72	82	1.0004	0.8371
2	101	300	0.9521	0.9145
3	82	100	0.9241	0.6149
4	165	74	1.0270	0.6268
Average	105	140	0.9761	0.7758

Residual solids of plant juice---------- 0.1016 g/500 ml

Net weight of yeast cell mass (0.7758-0.1016) g--------- 0.6742 g/500 ml

Weight of cell mass recovered from SDB---------- 0.9761 g/500 ml

Comparative recovery of yeast cells from plant juice

(24 hours)---------- 70%

Table II

Comparison Of Dry Weight Of Yeast Cell Mass After 48 Hours Of Incubation In Sabouraud Dextrose Broth And Plant Juice

Sample	Cell Density $1x10^6$		Dry Weight of Cell Mass (g)	
	Control	Plant Juice	Control	Plant Juice
1	83	37	1.0411	0.8491
2	56	6	1.3025	0.9219
3	52	12	1.2950	0.6162
4	15	121	1.1284	0.8373
Average	52	44	1.1917	0.8304

Residual solids of plant juice---------- 0.1016 g/500 ml

Net weight of yeast mass (0.8304-0.1016) g---------- 0.7288 g/500 ml

Weight of cell mass recovered from SDB---------- 1.1917 g/500 ml

Comparative recovery of yeast cells from plant juice

(48 hours)---------- 61%

Table III

Per Cent Of Oxalic Acid Concentrations In Water Hyacinths

Sample	Leaf (%)	Petiole (%)	Root (%)
1	0.6702	0.2529	0.1159
2	0.7885	0.3199	0.1567
3	0.8480	0.2901	0.1027
4	0.7885	0.2204	0.1701
Average	0.7738	0.2714	0.1360

The oxalic acid level in fresh water hyacinth as a whole is 0.3935 % while the oxalic acid concentration in dry water hyacinth as a whole is 6.0-9.1 %.

7. Oxalic Acid Level of Water Hyacinth

The calculation of the oxalic acid level in the sample was as follows:

Net weight of water hyacinth---------- A g

Weight of water hyacinth slurry---------- B g

Volume of 0.01 N $KMnO_4$ solution needed to titrate blank---------- C ml

Volume of 0.01 N $KMnO_4$ solution needed to titrate sample---------- D ml

$$0.45 \times ((30/20) \times (500/25)) \times 100 = 1350$$

$$\text{mg of oxalic acid per 100 g of water hyacinth} = \frac{(D-C)\ \text{ml} \times 1350 \times (A+100)}{A \times B}$$

whereas

0.45 = mg of anhydrous oxalic acid equivalent to 1 ml 0.01 N $KMnO_4$

100 = to convert to 100 g product

30/20, 500/25 : dilution factors

The comparative levels of oxalic acid in leaf, petiole and root are shown in Table III. The highest oxalic acid concentration occurred in the leaf portion whereas the root contained the lowest concentration. The petiole consisted of an average level of oxalic acid. This result was corresponding to the finding of Rao and his associates. They discovered that the leaf had the highest concentration of calcium which was associated with the oxalic acid level because oxalic acid was in the form of calcium oxalate usually. In roots, the calcium concentration was also found to be the lowest. The amount of accumulation of oxalate depends upon the

age of the plant. Normally when a plant is fully matured, its leaves will accumulate the highest level of oxalate. According to James, this figure lies below the critical oxalate level which was found to be 10 % or more on a dry weight base.

Some plants have a relatively high level of oxalic acid which may be due to the activity of carbohydrate metabolism, interconversion of components in the tricarboxylic acid cycle, glycolate metabolism, pyruvic acid metabolism or conversion of L-ascorbic acid.

Once oxalic acid was consumed by a ruminant, it might

i. be degraded by rumen flora;

ii. combine chemically with calcium to become insoluble and therefore unavailable for absorption from the rumen; or

iii. be absorbed from the rumen into the blood stream where it could combine with calcium to produce a hypocalcemia and interfere with other body processes.

The calcium oxalate crystals may precipitate out in various tissues, especially the kidneys. Hypocalcemia and uremia in the third case are responsible for the death of the animal. However, there is evidence that other factors may be involved in oxalate death. These include hemorrhagic rumenitis and shock, interference with energy metabolism (oxalate competitively inhibits enzymatic oxidation of lactate, interferes noncompetitively with reduction of pyruvic acid and inhibits succinic dehydrogenase), and cardiovascular collapse of depression of the central nervous system.

8. Uptake of Escherichia coli by Roots of Water Hyacinths

The number of bacteria in the control group (phosphate buffer in

distilled water) declined very rapidly during the first two days probably due to the fact that the solution in which the organism grew was only buffered by phosphate in distilled water (Fig. 6). It would have been more advantageous to use physiological saline but the plant is unable to tolerate a 0.85 % NaCl concentration.

The number of bacteria in plant roots and the water in which the plants were submerged, respectively, increased tremendously on the first day and then decreased slightly on the second, third and fourth day. The original average number of bacteria in roots was $40x10^3$ per gram of wet weight of plant roots.

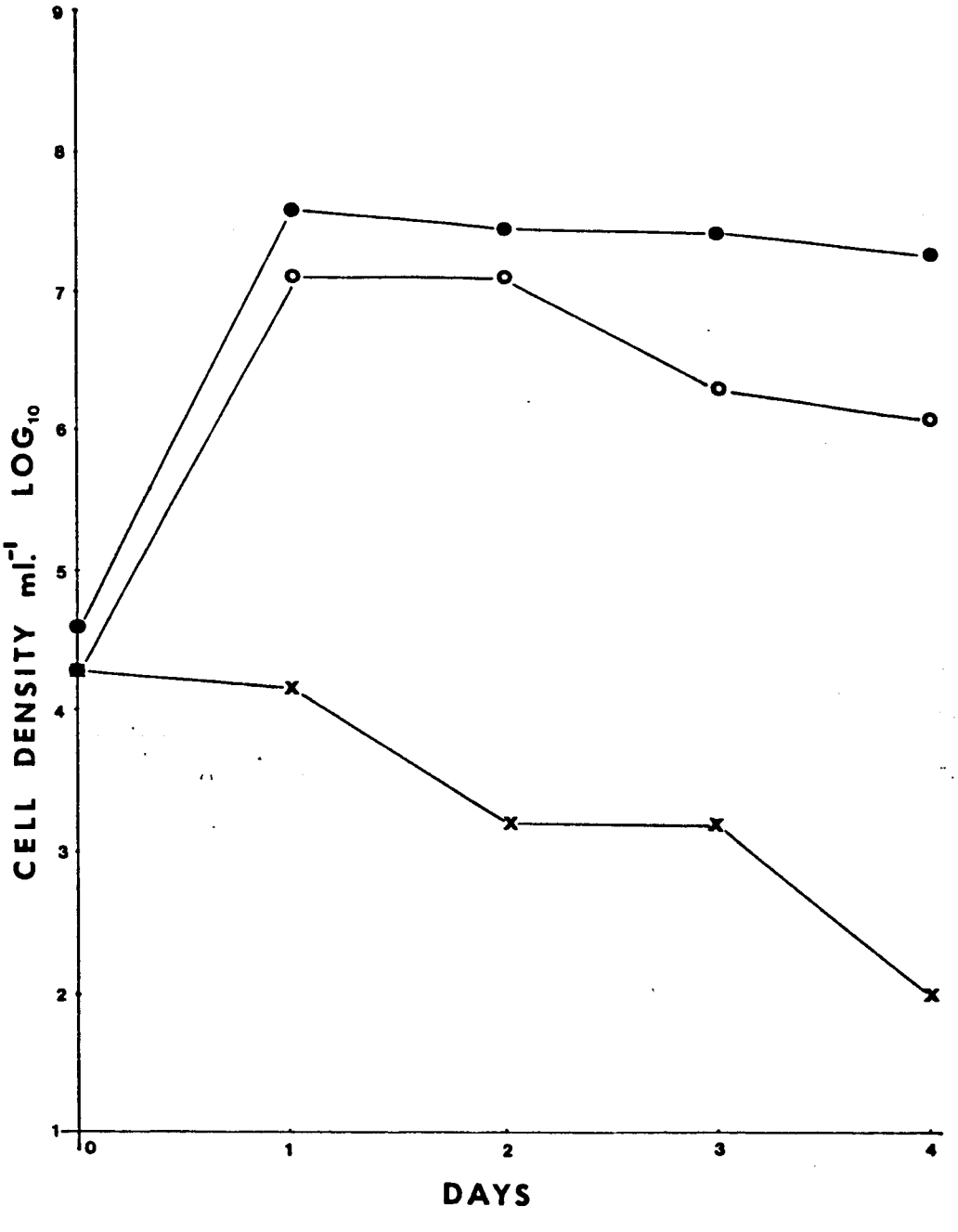
9. Uptake of Poliovirus by Roots of Water Hyacinths

Analyses of water samples of the control group as compared to the experimental group revealed that the number of Poliovirus from Day 0 to Day 4 remained constant, indicating that the plant was unable to absorb this particular virus. However, other types of viruses, such as mouse encephalomyelitis, were observed to be absorbed and even translocated to the aerial portion of the plant.

10. Antibiotic Activity of Water Hyacinths

In the antibacterial activity test a positive result will give a clear zone of inhibition around the loaded discs. This zone of inhibition is caused by the diffusion of antibiotics from the loaded disc that inhibits the growth of microorganism immediately around the disc. In this study, no anti-microbial activity could be observed in extracts from roots and

Figure 6. Uptake of *Escherichia coli* by Roots of Water Hyacinths


CELL DENSITY ml.$^{-1}$ LOG$_{10}$
9
8
7
6
5
4
3
2
1
0
1
2
3
4
DAYS
x—x control
o—o Plant water
●—● " root

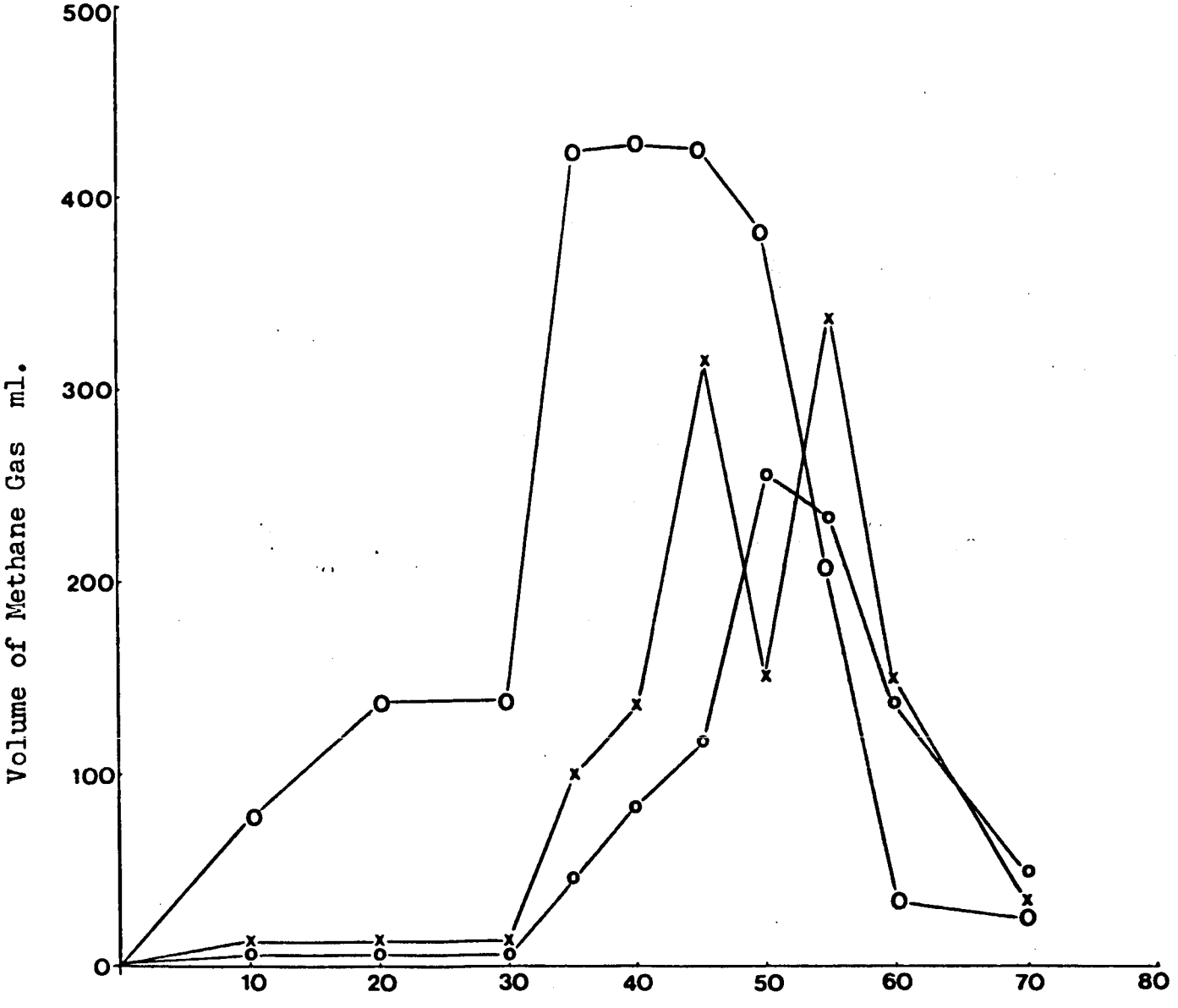
the green parts (petiole and leaf) though every kind of precaution was taken to avoid the destruction or loss of antibiotics, if any, in water hyacinth. These measures included condensing plant extracts to a very concentrated status (reduced volume from 200 ml to 2 ml) by evaporation at low temperature (37^{0} C). The former precaution served to create an effective dosage while the latter served to preserve the antibiotic activity of the extract. Since there was no antibiotic activity indicated in these crude extracts, a further purification of the plant extract was carried out according to methods described by Majumdar. But this experiment also failed to reveal any anti-microbial activity.

When we analyzed the body of water in which water hyacinth are submerged, we find that there were numerous types of microorganisms, plants and animals (including parasites) living around it. A total count of bacteria in the roots of water hyacinth revealed millions and millions of microorganisms living in the root meshes. A kind of nematode (parasite) is observed living on the rhizome of water hyacinth externally. According to Chitwood, Seinhorst and Starkey plant nematodes feed mainly on liquid contents of plant cells, fungi, protozoa and bacteria. This observation may not be important but it may be an indication of its inability to produce any antibiotic substance.

11. Methane Gas Production from Plant Residues of Water Hyacinths

The total volume of methane gas produced by the plant was about 2300 ml/250 g of plant during total time of incubation (Fig. 7). The production of gas terminated on the 60th day. The total volume of gas produced by plant residues (plant minus plant juice) was approximately 900 ml/250 g. The gas production terminated on the 70th day. The total

Figure 7. Methane Gas Production from Plant Residues of Water Hyacinths



O——O plant residues with plant juice

o——o plant residues without plant juice

x——x heat treated plant residues without plant juice

volume of methane gas produced by plant residues (plant juice removed after heating) was approximately 1100 ml/250 g. The production of gas ceased on the 70th day. In the last two experiments, maximum activity was achieved approximately one and half months after inoculation. The volume of methane gas produced by the entire plant was almost twice as much as the volume of gas produced from plant residue alone in the absence of plant juices.

However, if one considers the fact that the extracted plant juices can well be used for the production of "single cell protein", the loss of methane gas somehow seems to lose its significance.